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TITLE:

Investigating Target Gene Function in a CD40 Agonistic Antibody-induced Colitis Model Using CRISPR/Cas9-based Technologies

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KEYWORDS:

CRISPR, CD40, colitis, immune system, in vivo, LSK

SUMMARY:

Here, we describe the methodology to knock out a gene of interest in the immune system using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease (Cas9)-based technologies and the evaluation of these mice in a cluster of differentiation 40 (CD40) agonistic antibody-induced colitis model.

ABSTRACT:

The immune system functions to defend humans against foreign invaders such as bacteria and viruses. However, disorders of the immune system may lead to autoimmunity, inflammatory disease, and cancer. The inflammatory bowel diseases (IBD)—Crohn's disease (CD) and ulcerative colitis (UC)—are chronic diseases marked by relapsing intestinal inflammation. Although IBD is most prevalent in Western countries (1 in 1,000), incident rates are increasing around the world. Through association studies, researchers have linked hundreds of genes to the pathology of IBD. However, the elaborate pathology behind IBD and the high number of potential genes pose significant challenges in finding the best therapeutic targets. Additionally, the tools needed to functionally characterize each genetic association introduce many rate-limiting factors such as

the generation of genetically modified mice for each gene. To investigate the therapeutic potential of target genes, a model system has been developed using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease (Cas9)-based technologies and a cluster of differentiation 40 (CD40) agonistic antibody. The present study shows that CRISPR/Cas9-mediated editing in the immune system can be used to investigate the impact of genes in vivo. Limited to the hematopoietic compartment, this approach reliably edits the resulting reconstituted immune system. CRISPR/Cas9-edited mice are generated faster and are far less expensive than traditional genetically modified mice. Furthermore, CRISPR/Cas9 editing of mice has significant scientific advantages compared to generating and breeding genetically modified mice such as the ability to evaluate targets that are embryonic lethal. Using CD40 as a model target in the CD40 agonistic antibody-induced colitis model, this study demonstrates the feasibility of this approach.

INTRODUCTION:

Autoimmune diseases refer to conditions in which a patient's immune system attacks their own cells and organs, resulting in chronic inflammation and tissue damage. Nearly 100 different types of autoimmune conditions have been described to date, affecting 3–5% of the human population[1]. Many of the autoimmune conditions, including systemic lupus erythematosus and IBD, lack effective treatments and present significant unmet medical needs. Currently affecting around 1.5 million people in the USA alone, IBD is a devastating disease marked by progressive, persistent, and relapsing intestinal inflammation with no available cure. Unraveling the underlying pathogenesis and pathophysiology is needed to deliver the novel treatment and prevention strategies that IBD patients require[2,3].

Over 230 different IBD loci have been identified through genome-wide association analyses (GWAS)[4]. Although these associations have elucidated new genes that are potentially important players in the key mechanisms and pathways of IBD, only a few genes from these loci have been studied. Some genes have been implicated in specific pathways. For example, the microbe-sensing pathway has been linked to nucleotide-binding oligomerization domain-containing protein 2 (NOD2); the autophagy pathway has been linked to autophagy-related 16 like 1 (ATG16L1), immunity-related GTPase family M (IRGM), and caspase recruitment domain family member 9 (CARD9); and the pro-inflammatory pathway has been linked to interleukin (IL)-23-driven T-cell responses[4]. Various in vivo mouse models have been used to functionally characterize genes identified through GWAS[5,6].

One of the key models used to study IBD pathogenesis[7,8] is the CD40 model of colitis, which induces innate immune intestinal inflammation following the injection of a CD40 agonistic antibody into immunodeficient (T and B-cell) mice. Primarily used to examine the contribution of innate immunity to IBD development, mostly macrophages and dendritic cells[9], it is unclear if disease can be induced in fully immune-competent wild-type (WT) mice. In addition to animal models, gene-specific tools are also required for the functional characterization of a gene, including chemical compounds and biologics. More importantly, genetically modified animals are

essential in revealing the function of a specific gene. However, the strategies typically used to make genetically modified mice—embryo injection and breeding—often take over a year and incur a significant financial cost. This rate-limiting process presents a significant challenge in the quest to elucidate the functions of the IBD-related genes identified by GWAS.

The protocol presented here provides a viable alternative to breeding genetically modified mice. First, as shown in the **Figure 1** schematic, lineage-negative, stem cell antigen1-positive, receptor tyrosine kinase Kit-positive (lineage-Sca1+c-Kit+ or LSK) cells are isolated from the bone marrow of Cas9 knockin (KI) mice bearing a specific allele (CD45.2) to allow donor immune cell tracking. Next, these cells are exposed to lentiviruses bearing different guide RNAs (gRNAs) and a fluorescent marker, violet-excited green fluorescent protein (VexGFP), to allow tracking of transduced cells. Two days later, VexGFP+ cells are sorted and injected into lethally irradiated recipient Ly5.1 Pep Boy mice, which are C57Bl/6 mice bearing the CD45.1 allele to allow for recipient immune cell tracking. Twelve weeks later, the immune system is fully reconstituted, and the mice can be enrolled into in vivo models.

In addition to the benefit of cost savings and faster time-to-generation compared to the generation and breeding of genetically modified animals, this methodology is ideal for targets that are embryonic lethal, as it specifically targets the hematopoietic compartment. Furthermore, for targets where there are no tools available, such as an antibody, this system provides a feasible approach. In summary, to address the challenges described thus far, an in vivo CRISPR/Cas9-based genome editing platform was developed to expeditiously generate genetically modified animal models[10-14]. This study demonstrates that intestinal inflammation in WT C57Bl/6 mice can be induced by a CD40 agonistic antibody. CD40 is a key regulator of disease in this model and was therefore used as a model target to validate the CRISPR/Cas9-based knockout and loss of gene function.

PROTOCOL:

All animal experiments performed following this protocol must be approved by the respective Institutional Animal Care and Use Committee (IACUC). All procedures described here were approved by the AbbVie IACUC.

1. Generation of required lentiviruses and procurement of donor and recipient animals

NOTE: The **Table of Materials** includes source and order number details for all animals, instruments, and reagents used in this protocol.

1.1. Construct the plasmids using a lentiGuide-puro vector modified to VexGFP or mCherry.

1.1.1. Clone the scrambled non-targeting gRNA (SgNone) or CD40-targeting gRNA into the modified vector.

NOTE: In this study, the sequences for each gRNA were as follows: SgNone, CTATGATTGCAACTGTGCAG; SgCD40.1, AGCGAATCTCCCTGTTCCAC; SgCD40.2, GACAAACAGTACCTCCACGA; and SgCD40.3, ACGTAACACACTGCCCTAGA.

1.1.2. Produce the lentiviral particles as described previously[15].

1.1.2.1. Co-transfect the gRNA-encoding plasmids into 293T cells with VSV-G and pLEX packaging plasmids.

1.1.2.2. Change the culture medium after 18 h of transfection, and collect the supernatants containing the virus after 24–48 h following the medium change.

1.1.2.3. To evaluate CD40 gRNA efficiency, generate a CD40 stable 293T cell line by transfection of a pcDNA3.1 plasmid that encodes for CD40.

1.1.3. Transfect the cells using the described lentiviruses, and evaluate them by fluorescence-activated cell sorting (FACS) two weeks later.

NOTE: Cas9 KI mice were used as donor mice. Ensure that the Cas9 KI mice are on a C57Bl/6 background (and are expressing the CD45.2 allele) when using C57Bl/6-Ly5.1 Pep Boy mice (expressing the CD45.1 allele) as recipients, so that donor and recipient cells can be tracked.

1.2. Design the experiment. For gene knockout (KO), use 3–6 gRNAs per gene. For each gRNA, prepare 20% extra recipient mice to account for animal loss during and after transplantation.

NOTE: The CD40 agonistic antibody-induced colitis model requires a minimum of 8 mice per group for statistical powering. Hence, 10 mice per gRNA were prepared in this study. The number of mice needed for other in vivo models will vary based on the model used.

1.3. Use same sex donor and recipient mice that are 8–12 weeks old.

2. Bone marrow harvest and preparation for cell sorting

2.1. Using forceps and scissors, harvest bones from each donor mouse (femur, tibia, humerus, and ulna), carefully removing as much muscle/tissue as possible.

2.2. Puncture the bottom of a 0.6 mL microcentrifuge tube with a 23 G needle, and place the tube inside a 1.5 mL centrifuge tube. Make 2 sets per animal.

2.3. Cut one end of the bones open, and place 4–8 bones inside the microcentrifuge tube with open ends facing toward the 23 G needle hole.

NOTE: The number of bones per tube depends on the bone, e.g., 8 tibiae, humeri, and ulnae or 4 femurs will fit.

2.4. Centrifuge the tubes (with the 0.6 mL microcentrifuge tube containing the bones inside the 1.5 mL centrifuge tube) at 300 × *g* for 2 min at room temperature (RT).

2.5. Discard the 0.6 mL microcentrifuge tube, now containing bones with no marrow, leaving just the 1.5 mL centrifuge tube now full of marrow. Add 1 mL of red blood cell lysis buffer (containing ammonium chloride) to each centrifuge tube, and resuspend the cell pellet by pipetting. Incubate at RT for 1 min. Repeat the lysis step if needed.

2.6. Transfer the cell suspension into a 50 mL conical tube, and add Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (at least double the volume of the cell suspension) to neutralize the lysis buffer. Pellet cells at 300 × *g* for 5 min. Aspirate the supernatant completely.

2.7. Load a 70 µm filter on a 50 mL conical tube, and filter the cells through, using DPBS to wash the tube and filter. Discard the filter, and count the cells.

NOTE: Using 30 donor mice (12 weeks old) will yield 200–300 × 10^6 cells.

3. Cell sorting to isolate LSK cells for transduction

3.1. Re-suspend the cells counted in step 2.7 in magnetic cell sorting (MACS) buffer (DPBS, 2 mM ethylenediamine tetraacetic acid, 0.5% bovine serum albumin, 10 µg/mL penicillin-streptomycin) in the desired volume (90 µL per 10^7 cells).

3.2. Add 10 µL of CD117+ beads per 10^7 cells. Mix well, protect from light, and incubate at 4 °C for 15 min.

3.3. Prepare the MACS LS column by placing the column in the magnetic field and rinsing it with MACS buffer.

3.4. Wash the cells from step 3.2 with an appropriate volume of MACS buffer (at least double your volume), and centrifuge at 300 × *g* for 10 min.

3.5. Aspirate the supernatant completely. Re-suspend the pellet so that the final concentration is 10^8 cells in 500 µL of MACS buffer.

3.6. Apply the cell suspension onto the LS column. Wash with 3 x 3 mL of MACS buffer.

NOTE: One LS column can take up to 10^8 labeled cells and 2 × 10^9 total cells.

3.7. Remove the column from the magnetic separator, and place it in a suitable collection tube. Add 5 mL of MACS buffer, and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

3.8. Pellet the cells at 300 × *g* for 10 min. Aspirate the supernatant completely.

3.9. Re-suspend the cells in 100 µL of MACS buffer per 10^7 cells, and add the staining antibodies for lineage (CD3, B220, Ter119, Gr-1, CD11b) and Sca-1.

NOTE: Using 30 donor mice (12 weeks old) should yield 100–180 × 10^6 cells.

3.10. Mix well, protect from light, and incubate at 4 °C for 20 min.

3.11. Wash the cells by adding 5–10 mL of MACS buffer, and centrifuge at 300 × *g* for 10 min. Aspirate the supernatant completely.

3.12. Re-suspend the cells to obtain 10^8 cells in 500 µL of MACS buffer

3.13. Filter the cells with a 70 µm cell strainer, and perform FACS for lineage-Sca1+ population.

NOTE: Approximately 1.8–2.6% of the population should be lineage-Sca1+.

3.14. Collect the cells in LSK culture medium (100 µL per 10,000 cells): serum-free expansion medium, 100 ng/mL thrombopoietin, mouse stem cell factor, Fms-related tyrosine kinase 3 ligand, and IL-7 with 100 µg/mL penicillin-streptomycin.

3.15. Pellet cells at 300 × *g* for 10 min. Aspirate the supernatant completely.

4. LSK transduction and culture to generate control and knockout cells

4.1. Resuspend the cells in LSK culture medium.

4.2. Seed 10,000 cells per well in 96-well flat-bottom tissue culture (TC) plates in LSK culture medium.

4.3. Incubate overnight at 37 °C with 5% CO_2 and 95% humidity under aseptic conditions.

4.4. Prepare 50 µg/mL retronectin solution in DPBS, and add 300 µL to each well of a non-TC-treated 24-well polystyrene plate. Incubate overnight at 4 °C.

4.5. The next day, discard the retronectin solution, rinse the coated plate with 300 µL of DPBS, and repeat.

4.6. Transfer 50,000 LSK cells in 500 µL of medium (5 wells from the 96-well plate in step 4.2) to each retronectin-coated well of the 24-well polystyrene plate from step 4.4. Use an additional 100 µL of LSK medium per 5 wells to rinse and collect any extra cells remaining in the 96-well

plate. Ensure that 600 µL is the volume per well in the 24-well plate following this step (5 × 100 µL wells from the 96-well plate + 100 µL used to wash those 5 wells).

NOTE: After transferring, look under a microscope to confirm that all cells were collected. Use LSK medium to collect cells that were not transferred to minimize cell loss from this small population.

4.7. Add 300 µL of virus supernatant to each well, and shake the plate at a setting value of 500 for 5 min. Spin the plate at 600 × *g* and 37 °C for 20 min. Use at least 5 × 10^6 viral particles per mL.

NOTE: In this study, a vector modified from pLentiPuro was used, in which the puromycin resistance element was swapped to VexGFP. The virus was added at a multiplicity of infection of 50–100.

4.8. Incubate for 1 h. Add 500 µL of pre-warmed LSK medium.

4.9. Incubate for 2 days at 37 °C with 5% CO_2 and 95% humidity under aseptic conditions.

5. Animal irradiation to prepare for donor stem cell engraftment

5.1. After 2 days of incubation, irradiate the recipient animals with 475 cGy twice at an interval of 4 h. Following the second round of irradiation, place the animals in autoclaved cages, and treat them as immunodeficient animals for 12 weeks.

NOTE. The irradiation dosages may differ with different irradiators. Perform a dose titration of the irradiator to identify the best dosage. Doses between 700 and 1300 cGy for C57Bl/6 mice are found to be effective in various literature examples[17]. Select 3–4 doses in this range, and evaluate 5 mice per dose for survival and engraftment. If engraftment is unsuccessful or the radiation dose is too high, mice will not live past 3 weeks post-engraftment. At 4 weeks post-engraftment, bleed the surviving mice to evaluate engraftment by FACS.

6. Cell preparation and injection into irradiated recipient animals

6.1. Pipet the cells out of each well from step 4.9, keeping the groups separate now that the cells have been transduced with different gRNAs, and pellet them at 300 × *g* for 10 min.

6.2. Resuspend the cells in MACS buffer, filter with a 70 µm strainer, and sort the VexGFP+ population.

NOTE: Approximately 10–15% of the cells should be VexGFP+.

6.3. Pellet the cells at 300 × *g* for 10 min. Resuspend them in Hank's balanced salt solution: 10,000 or more cells per mouse in 200 µL.

6.4. Inject the cells intravenously 3 h after the last dose of irradiation.

NOTE: Twelve weeks later, the mice will have a fully engrafted immune system and can be enrolled into in vivo models.

7. CD40 agonistic antibody-induced colitis model in wild-type mice

NOTE: Weigh and assess the animals daily. Provide supportive care as needed: 1.0 mL of subcutaneous sodium chloride solution at 10% weight loss or if they are dehydrated. The positive control for this model is anti-p40 dosed intraperitoneally at 25 mg/kg twice per week beginning on day -1.

NOTE: In this study, experimental groups included naïve control, vehicle (negative) control, and anti-p40 (positive) control groups. Together, these groups control for the normal behavior of the CD40 agonistic antibody-induced colitis model. Vector, SgNone, and SgRNA groups: The vector controls for the common lentiviral vector, SgNone is a scrambled non-targeting guide control, and the SgRNA groups are the "treatment" groups bearing reduced expression of the target gRNA.

7.1. Day 0: Inject CD40 agonistic antibody at 10 mg/kg intraperitoneally in DPBS into all animals except for naïve control.

7.2. Days 3 and 6: Perform video endoscopy to evaluate disease progression as previously described[16].

7.2.1. Anesthetize the mice with 2-3% isoflurane.

7.2.2. Once the mice are under anesthesia, administer DPBS (without calcium and magnesium) enema to prepare the colon for endoscopy.

NOTE: Approximately 1–2 mL is required to perform an enema.

7.2.3. After enema and while under anesthesia, move the mouse to the nose cone of the anesthesia machine.

7.2.4. Gently insert the endoscope into the colon, slowly advance to the proximal colon while keeping the camera centered and the colon inflated with air (using either the included air pump or attach a tube/syringe to manually inflate), then slowly withdraw the endoscope, and collect a video and images at 3 cm, 2 cm, and 1 cm from the anus.

7.2.5. Score each image individually based on the vascular pattern and thickening of the mucosa as described in **Table 1**. Combine the scores from the 3 images per mouse to assign a sum score to each animal.

7.3. Day 7: Euthanize all mice by isoflurane overdose or CO_2 chamber, and collect as much blood as possible by cardiac puncture for serum. Weigh the spleen for flow cytometry, and collect the colon for histopathology.

NOTE: Formalin-fixed and paraffin-embedded tissue sections of mouse colon were prepared and used for immunohistochemistry (IHC) as previously described by Wang et al[19].

REPRESENTATIVE RESULTS:

Following the procedure described above, mice expressing CD40-targeted gRNA were generated. By week 2, B-cells, CD11b+ macrophages, and CD11c+ dendritic cells (DCs) were engrafted (**Figure 2**). T-cells however, as expected based on previous literature[18], took longer to fully engraft and required 12 weeks post-engraftment to reach ~90% (**Figure 2**). Immune organs, such as the spleen and lymph nodes, had the most notable population of donor-derived cells; however, other organs including the liver, lung, and intestine also showed the presence of donor cells (**Figure 3**). A strong reduction in CD40 expression was observed only in mice expressing CD40-targeting gRNA (**Figure 4**).

Similar to immunodeficient mice, following the CD40 agonistic antibody injection, WT C57Bl/6J mice exhibited body weight loss, vascular loss, and mucosal thickening as observed by colonoscopy, and myeloid cell infiltration determined by IHC with ionized calcium-binding adaptor molecule 1 (IBA1) (**Figure 5**). In addition to the typical readouts for the CD40 agonistic antibody-induced colitis model, an adaptive immune response was also observed, as shown by CD3 IHC revealing T-cell infiltration in the colon and by FACS analyses revealing T and B cell activation through CD86 upregulation on splenocytes (**Figure 5**). Importantly, an anti-p40 monoclonal antibody inhibited disease induction (**Figure 5**), which was consistent with the findings with the model using immunodeficient strains[9].

A reduction in CD40 expression by targeted gRNA protected the mice from CD40 agonistic antibody-induced colitis. The degree of protection correlated with the editing efficiency of each gRNA (**Figure 6**). More specifically, SgCD40.1 was the most efficient gRNA (**Figure 4**), which led to the most powerful disease inhibition, as indicated by CD86 upregulation and intestinal infiltrate of immune cells (**Figure 6D,E**). Conversely, SgCD40.2 had the least editing efficiency of all three gRNAs used in vitro, which resulted in the least protection in vivo; SgCD40.3 exhibited intermediate editing and protection (**Figure 6D,E**). Taken together, the data shown here reveal the feasibility of using CRISPR/Cas9 to reduce the expression of a target, which can successfully protect from colitis induction. Most importantly, these results demonstrate that this in vivo CRISPR/Cas9-based platform can be used to investigate gene function in the pathogenesis of intestinal inflammation.

FIGURE AND TABLE LEGENDS:

Figure 1: Stem cell harvest, transduction, and transplantation strategy used to generate CRISPR/Cas9-edited mice. Bones are harvested from Cas9 KI mice, bearing the CD45.2 allele as a donor cell marker. Bone marrow is isolated, and the cells are sorted for Lin-Sca1+c-Kit+ (LSK) stem cells. These cells are then exposed to various lentiviruses (vector control, SgNone Control, SgCD40.1), all bearing VexGFP as a fluorescent marker to indicate transduced. Stem cells are then sorted for VexGFP+ cells to inject a pure population of edited stem cells into lethally irradiated recipient mice. The recipient mice are Ly5.1 Pep Boy mice, which are C57Bl/6 WT mice bearing the CD45.1 allele as a recipient cell marker. Abbreviations: KI = knockin; VexGFP = violet-excited green fluorescent protein; WT = wild-type.

Figure 2: Differentiation of immune cell subsets after LSK transplantation. CD45.2+ donor mice were euthanized, and their LSK cells were isolated and transplanted into lethally irradiated CD45.1 congenic C57Bl/6 animals. Every other week following transplantation, a small cohort of mice (n=5) was euthanized, and the engraftment rates in the spleen, bone marrow, and blood were evaluated by FACS. The percentage of donor cell subsets at different timepoints post-transplantation is shown. Each dot represents a data point from a single animal. Abbreviations: LSK = Lin-Sca1+c-Kit+; FACS = fluorescence-activated cell sorting; DC = dendritic cell; SEM = standard error of the mean.

Figure 3: Distribution of donor LSK-differentiated cells in reconstituted animals. Recipient animals were reconstituted using LSK cells infected with mCherry-expressing virus. Tissues were harvested at week 12 post-transplantation and mCherry expression (brown) was evaluated by IHC: (**A**) spleen, (**B**) mesenteric lymph nodes, (**C**) lung, (**D**) liver, (**E**) small intestine, (**F**) large intestine, (**G**) kidney, (**H**) skin. This figure has been modified from Wang et al.[19]. Scale bar = 100 µm. Abbreviations: LSK = Lin-Sca1+c-Kit+; IHC = immunohistochemistry.

Figure 4: CRISPR/Cas9-mediated reduction in CD40 expression on B-cells. CD40 expression reduction in reconstituted mice, modulated using a CRISPR/Cas9-based platform. Splenocytes were evaluated 8 weeks post-transplantation by FACS for CD40 expression on B-cells. Each dot represents an individual animal. *$p<0.005$ Data shown are representative of two independent experiments. Abbreviations: FACS = fluorescence-activated cell sorting; SEM = standard error of the mean; Sg = single guide RNA.

Figure 5: CD40 agonistic antibody-induced intestinal inflammation in C57Bl/6 mice. CD40 agonistic antibody was injected into C57Bl/6 mice to induce inflammation, and disease induction was evaluated based on (**A**) body weight change, (**B**) colonoscopy at day 3 and day 6 post-CD40 agonistic antibody injection, (**C**) percentage of IBA1+ and CD3+ areas of total mucosal area, mucosal thickness, as well as percentage of goblet cell area of total mucosal area, and (**D**) upregulation of CD86 expression in splenic B and T cells. In (**B**), representative images for day 6 colonoscopy are shown. In (**C**), representative images for day 7 histology are shown. Scale bar = 100 µm. *$p<0.001$ Data are representative results from two independent experiments. This figure has been modified from Wang et al[19]. Abbreviations: IBA1 = ionized calcium-binding adaptor molecule 1; SEM = standard error of the mean.

Figure 6: CRISPR/Cas9-based knockout of CD40 ameliorates disease pathogenesis in a CD40 agonistic antibody-induced colitis model. LSK cells were infected with lentivirus-expressing control or SgCD40 and sorted for VexGFP. VexGFP+ cells were used to transplant lethally irradiated CD45.1+ recipient mice (n=10). Twelve weeks post-transplantation, colitis was induced by injecting CD40 agonistic antibody. (**A–D**) Disease induction was assessed by (**A**) body weight change, (**B**) colonoscopy at day 3 and day 6 post-CD40 agonistic antibody injection, (**C**) percentage of IBA1+ and CD3+ cell areas of total mucosal area, mucosal thickness as well as percent of goblet cell area of total mucosal area, and (**D**) upregulation of CD86 expression in splenic B and T cells. Representative (**B**) day 6 colonoscopy images and (**C**) day 7 histology images are included. Each dot represents an individual animal. Scale bar = 100 μm. *p<0.001. Data shown are representative of two experiments. This figure has been modified from Wang et al[19]. Abbreviations: IBA1 = ionized calcium-binding adaptor molecule 1; LSK = Lin-Sca1+c-Kit+; VexGFP = violet-excited green fluorescent protein; Sg = single guide RNA; SEM = standard error of the mean.

Table 1: CD40 agonistic antibody-induced colitis endoscopy scoring scale. Images of the colon were collected at 3 cm, 2 cm, and 1 cm from the anus. Each image was then evaluated for vascularity and thickening, scoring each parameter from 0–3 as indicated. The total score from all 3 images per animal was then combined to assign a sum endoscopy score to each animal.

DISCUSSION:

The results shown here introduce a novel CRISPR/Cas9-based genome editing platform capable of investigating gene function in this CD40 agonistic antibody-induced colitis model. Cell sorting enriched the pool of genetically modified LSK cells, resulting in over 90% reduction in CD40 expression within the reconstituted animals—in just 4 months. Furthermore, the reduced expression of CD40 within the immune system had a profound effect within the CD40 agonistic antibody-induced colitis model, significantly reducing disease endpoints. Based on these results, an in vivo CRISPR/Cas9-based platform was established, which provides researchers with a powerful tool to study a gene's function within the immune system. This technological advancement will expedite the process of validating new target biology, and ultimately, the ability to deliver transformative therapies to IBD and autoimmune patients in need.

The CRISPR/Cas9-based platform presented here enables efficient and efficacious gene modulation in WT C57Bl/6 mice. As the platform utilizes lethal irradiation and bone marrow transplants, it is expected that animals will be lost prior to complete engraftment. Therefore, powering the groups by 20% extra mice will help to account for these losses. As LSK cells isolated after 5-fluorouracil treatment had reduced c-kit expression, cell sorting is recommended to isolate LSKs. Additionally, high-titer virus should be used after concentration via ultracentrifugation. However, despite the high titer, the viruses did not efficiently infect LSK cells in this study. Perhaps ultracentrifugation concentrated the inhibitory factors, necessitating the use of a sucrose gradient to improve efficiency.

Usually the CD40 agonistic antibody-induced colitis model is used with immunodeficient recombination-activating gene (RAG) and severe combined immunodeficient (SCID) mice, as the primary value in the model is to evaluate innate immunity rather than adaptive immunity. Induced mice exhibit body weight loss, splenomegaly, intestinal inflammation, and myeloid cell infiltration in the colon. Demonstrated here, wild-type C57Bl/6 mice respond similarly to the CD40 agonistic antibody and to the positive control, anti-p40, which inhibited disease. The two main differences between the WT C57Bl/6 mice and immunodeficient mice are 1) WT C57Bl/6 mice require twice the dose of the CD40 agonistic antibody and 2) WT C57Bl/6 mice can have reduced disease levels on day 6 compared to day 3, as measured by endoscopy. Generally, immunodeficient mice maintain a similar level of disease from day 3 to day 6, sometimes showing exacerbation. More experiments are needed to definitively elucidate the cause of this difference, but the hypothesis points to regulatory T-cells, which are not present in immunodeficient mice, limiting and/or reversing disease a week after CD40 agonism.

In conclusion, this in vivo CRISPR/Cas9-based platform utilizes and combines LSK transplantation with CRISPR gene editing to efficiently reduce the expression of target genes within the immune system. With the potential to edit multiple genes in LSK cells via CRISPR/Cas9 in concert, this platform may provide the opportunity to evaluate digenic or polygenic phenotypes seen in IBD patients. Critical to efficiently and effectively assessing genes linked to IBD patients, this platform expedites the ability to accurately evaluate the function of genes within the immune system, which will reduce the time from discovery to the development of life-changing therapeutics.

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DISCLOSURES:

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Figure 1

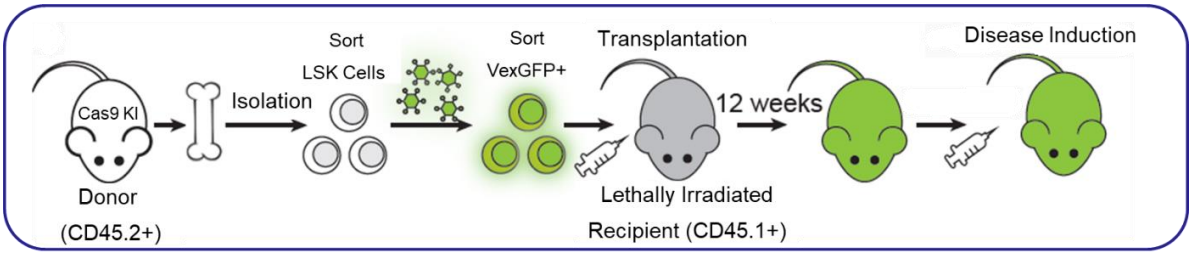

Figure 2

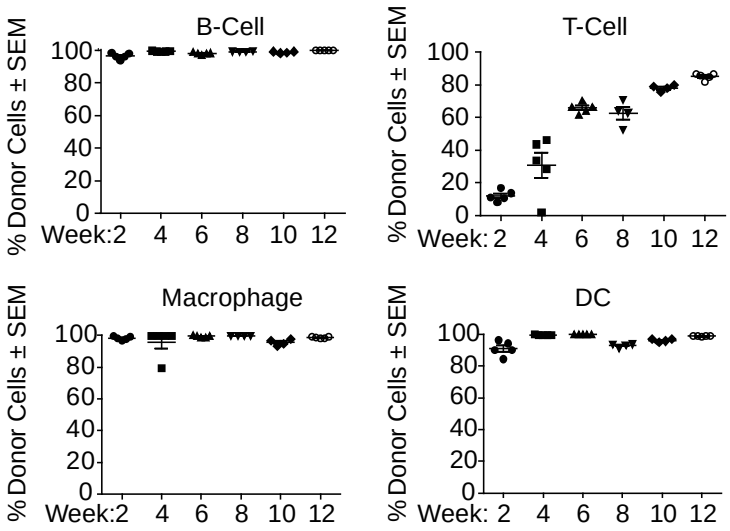

Figure 3

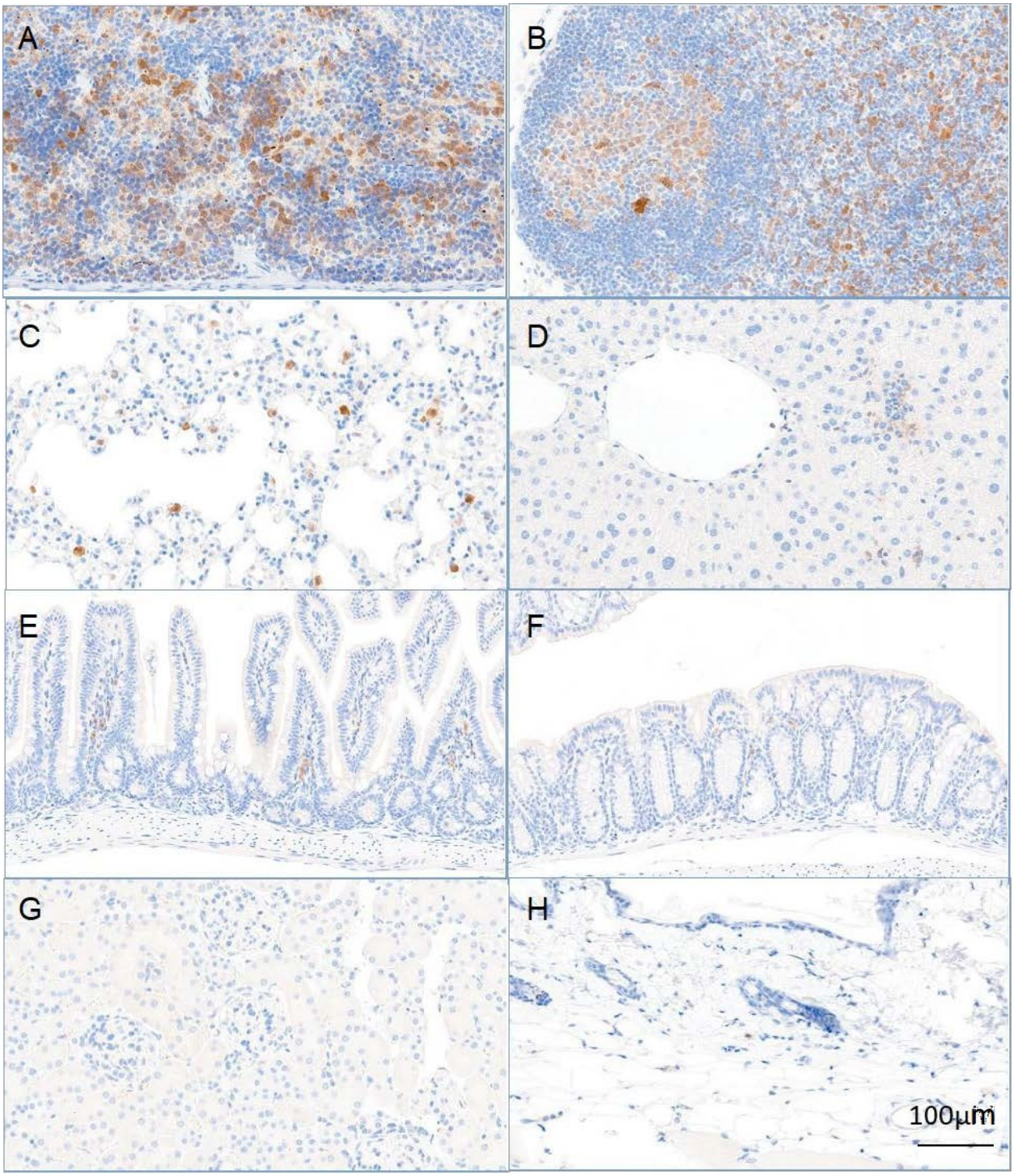

Figure 4

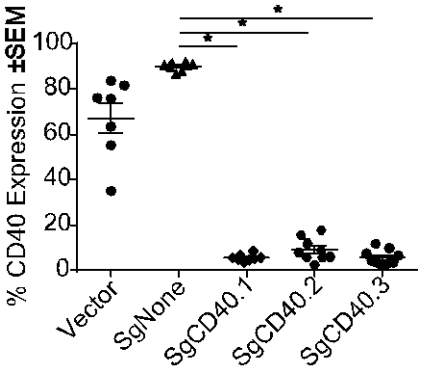

Figure 5

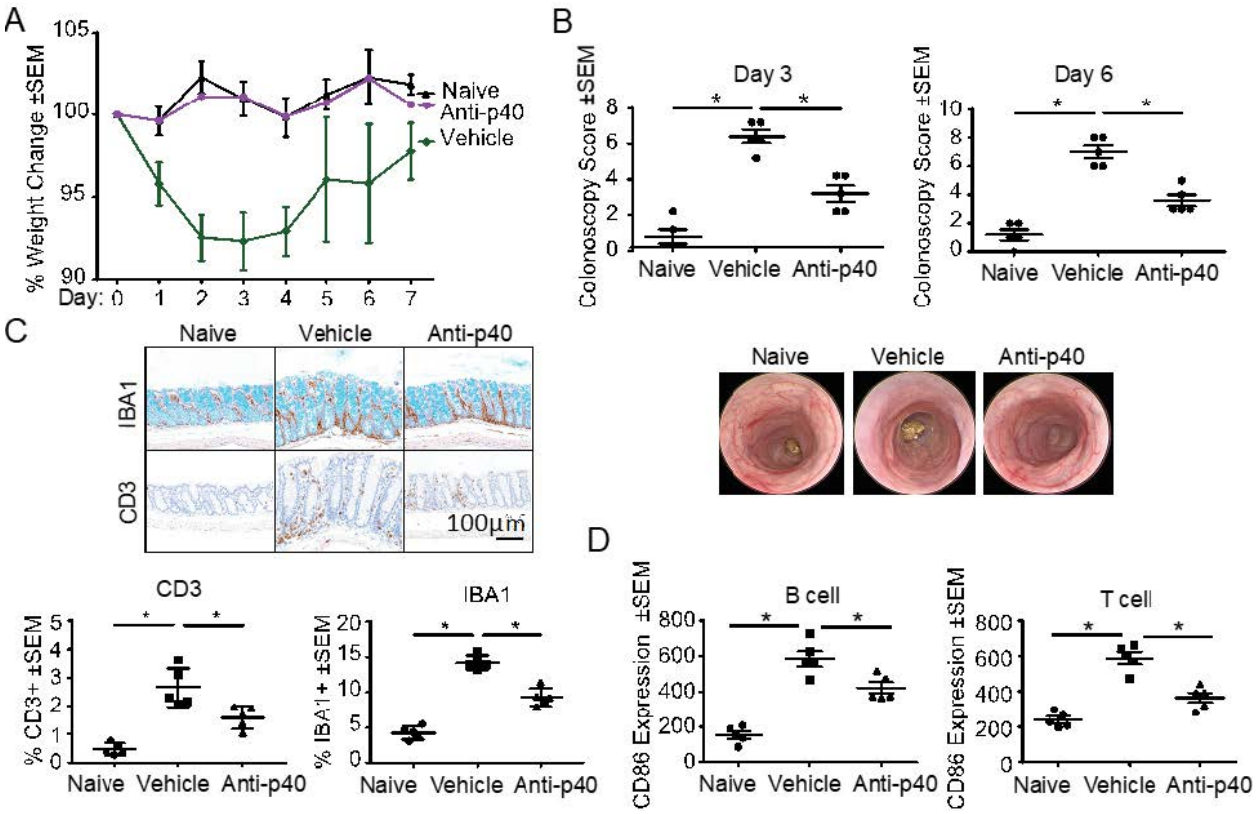

Figure 6.

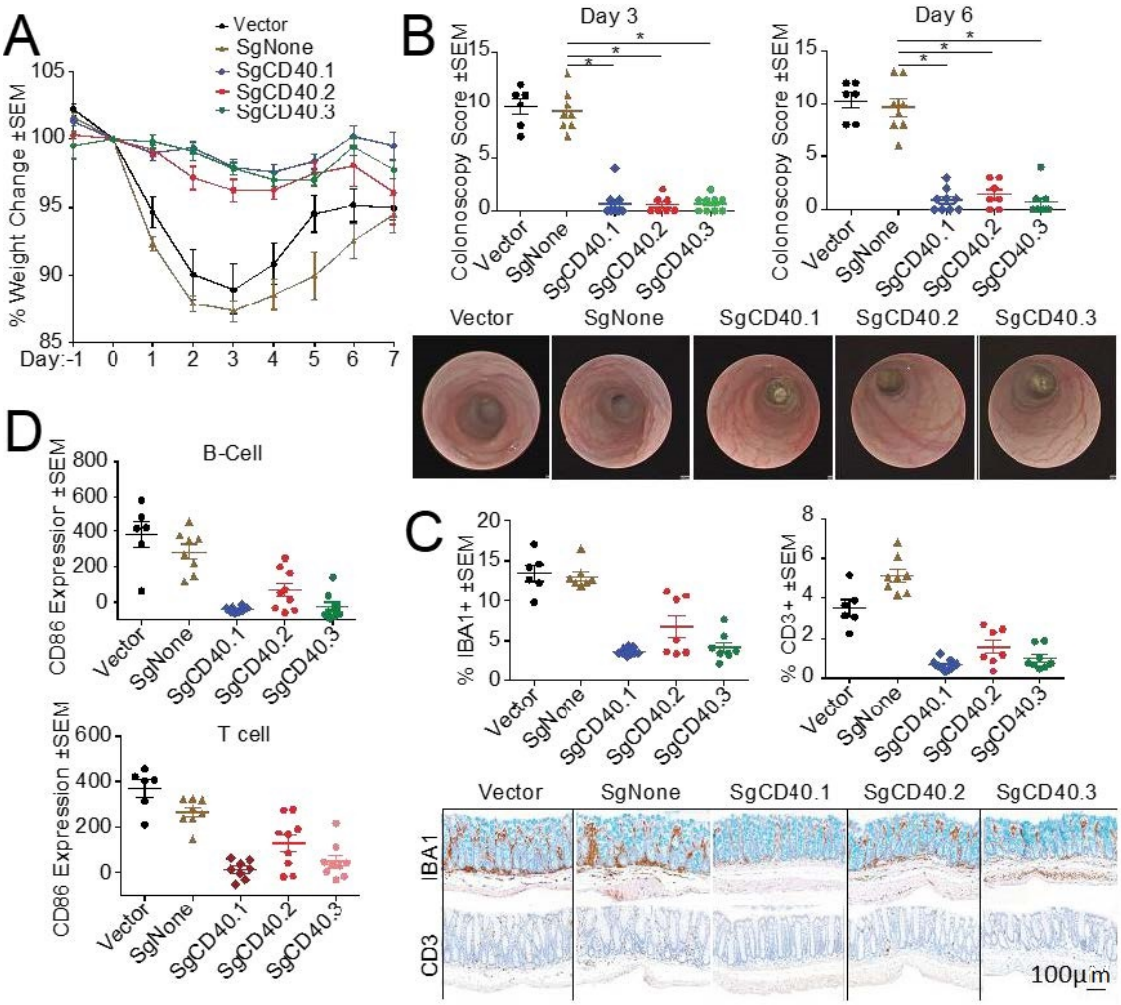

Table 1

Score	Vasculature	Thickening
0	The small and large blood vessels are bright, sharp, and have a continuous pattern,	The surface of the colon is smooth and shiny.
1	The small and large blood vessels are visible, but not connecting and out of focus.	The mucosal wall is less transparent and slightly bumpy with a shiny mucous layer.
2	The large blood vessels are still visible, but discontinuous, and a number of small vessels appear to have burst.	There is a clear white, shiny, bumpy layer covering most of the circumference.
3	No blood vessels are visible, and the surface of the colon is very bumpy.	There is an opaque, white, bumpy surface covering the circumference.

Name of Material/ Equipment	Company	Catalog Number
6-well tissue culture plates	Corning/Costar	#3506
TransIT-LT1	Mirus Bio	MIR 2300/5/6
MACS Buffer (autoMACS Running Buffer)	Miltenyi Biotec	130-091-221
0.45 µm filter unit	Millipore	#SLHV013SL
0.6 mL microcentrifuge Tube	Axygen	MCT-060-C-S
1.5 mL Eppendorf Tube	Axygen	MCT-150-C-S
15mL Conical	VWR	21008-918
23 G Needle	VWR	#305145
24 Well Non-TC Plates	Falcon	#351147
24-Well TC Plates	Falcon	#353047
50 mL Conical tube	VWR	21008-951
5 mL Syringe	BD Biosciences	#309647
70 µm Filter	Miltenyi	#130-098-462
96-Well Flat Bottom Plates	Corning	#3599
96-Well U-Bottom Plates	Corning/Costar	#3365
Anesthesia Machine	VetEquip - COMPAC5	#901812
Anti-CD40 Agonist monoclonal antibody	BioXcell	BE-0016
Anti-p40 monoclonal antibody	BioXcell	BE-0051
B220 PE Antibody	BioLegend	#103208
Bovine serum albumin	Sigma Aldrich	A7906-100G
Cas9 Knock-in Mice	Jackson Labs	#026179
CD117+ Beads	Miltenyi	#130-091-224
CD11b PE Antibody	BioLegend	#101208
CD3 PE Antibody	BD Biosciences	#553240
Centrifuge	Beckman Coulter	Allegra 6KR Centrifuge
Countertop Centrifuge	Eppendorf	Centrifuge 5424
DPBS	ThermoFisher	#14190136
Dulbecco’s Modified Eagle Medium	Mediatech	#10-013-CV
Ethylenediamine tetraacetic acid (EDTA)	Invitrogen	AM9260G
Endoscope	Karl Storz	N/A
Flow cytometer	BD Biosciences	FACS Aria II
Fms-related tyrosine kinase 3 ligand (Flt-L)	PeproTech	#250-31L

Gr-1 PE Antibody	BD Biosciences	#553128
Hank's balanced salt solution (HBSS)	ThermoFisher	#14170120
Heat-Inactivated Fetal Bovine Serum	HyClone	#SH30071.03
IL-7	PeproTech	#217-17
Incubator	Binder	#9040-0116
Isoflurane	HenrySchein	#6679401710
LS Column	Miltenyi	#130-042-041
Ly5.1 Pepboy Mice	Jackson Labs	#002014
mouse stem cell factor (mSCF)	PeproTech	#250-03
Sodium chloride (NaCl)	Hospira	#00409488850
OPTI-MEM serum-free media	Invitrogen	#31985-070
Penicillin-streptomycin (PenStrep)	ThermoFisher	#15140-122
Plate Shaker	ThermoFisher	#88880023
pLentiPuro	Addgene	#52963
Polybrene (10 μg/μL)	Sigma Aldrich	#TR-1003-G
Red Blood Cell Lysis Buffer	eBioscience	#00-4333
Retronectin	Takarbio	#T100B
Sca-1 APC Antibody	BioLegend	#108112
StemSpan	StemCell Technologies	#09600
Ter119 PE Antibody	eBioscience	#12-5921
Thrombopoietin (TPO)	PeproTech	#315-14
X-ray Irradiator	Precision X-Ray	X-Rad 320

Comments/Description

C57Bl/6 background

Custom Coloview Tower

C57Bl/6 background

Dear Dr. Nguyen:

Thank you very much for overseeing the review of our manuscript titled "**Investigating target gene function in a CD40 agonist-induced colitis model using CRISPR/Cas9-based technologies**." [JoVE61618R1]. We would also like to thank the reviewers for their positive and thoughtful comments and efforts towards improving our manuscript.

Enclosed please find revised version of our manuscript in which we have fully addressed all the comments/concerns raised by the reviewer. Attached are our detailed point-by-point responses to the reviewer's comments; our responses and the changes in the manuscript are both marked with blue color.

We hope you would agree that all the concerns raised by the reviewers have been appropriately addressed, and that all the changes in the current version of manuscript have substantially improved the quality and clarity of the manuscript. We further hope that, with incorporation of these changes, our manuscript will be suitable for publication in *JoVE*.

Please let us know if we can provide any additional information.

Sincerely,

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All authors are employees of AbbVie. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication. This does not alter our adherence to all JoVE policies on sharing data and materials.

We want to thank the reviewers for their overall positive response to our manuscript and for acknowledging the potential impact of our work on the application of CRISPR/Cas9-based technologies in studying the immune system. We appreciate that some shortcomings in our previous version of manuscript have been pointed out, and we have addressed those in the revised version of manuscript. We hope that the reviewers agree that the new manuscript has addressed all the comments. We also would like to thank the reviewers for their thoughtful comments and efforts towards improving our manuscript.

POINT-BY-POINT- REPLY to Reviewers' Comments on the manuscript "Investigating target gene function in a CD40 agonist-induced colitis model using CRISPR/Cas9-based technologies." (JoVE61618R1):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling and grammar. Please also check formatting, e.g., CO2 in 4.3.

Thank you for this note, we carefully went through the manuscript and made many changes to ensure spelling and grammar errors were corrected. For example, Line 164 Page 4, Section 2.6 was edited as shown below:

Previous: 2.6 Transfer cell suspension into conical tubes and add DPBS to neutralize the lysis buffer. Pellet cells at 300 x g for 5 minutes. Aspirate supernatant completely.

Current: 2.6 Transfer the cell suspension into a 50mL conical tube and add Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (at least double the volume of the cell suspension) to neutralize the lysis buffer. Pellet cells at 300 x g for 5 minutes. Aspirate supernatant completely.

2. In the abstract, please emphasize the method used (advantages, limitations, applications) rather than the results; please revise lines 43-45.

Great point. We added the following lines to describe the advantages, limitations, and applications - lines 44-51 on pages 1-2.

Previous: In the present study, we showed anti-CD40 agonist antibody induces intestinal inflammation in wild type animals and that a CD40 knockout in the immune system using CRISPR/Cas9 technologies inhibited disease development.

Current: In the present study, we showed CRISPR/Cas9-mediated editing in the immune system can be used to investigate the impact of genes in vivo. Limited to the hematopoietic compartment, this approach reliably edits the resulting reconstituted immune system. CRISPR/Cas9 edited mice are generated faster and are far less expensive than traditional genetically modified mice, and furthermore, have significant scientific advantages compared to generating and breeding genetically modified mice, such as the ability to evaluate targets that

are embryonic lethal. Using CD40 as a model target in the CD40 agonistic antibody induced colitis model, we demonstrated the feasibility of this approach.

3. Throughout the manuscript, please consider saying either anti-CD40 antibody or CD40 agonistic antibody to avoid confusion.

Thank you for pointing out this inconsistency. We change it to CD40 agonistic antibody throughout, beginning on line 28 page 1 in the summary section.

4. In the Introduction, please include information to help readers to determine whether the method is appropriate for their application.

We added the following text to the introduction to better describe the circumstances that may drive a researcher to use this method. Lines 92-96 on page 3.

In addition to the benefit of cost savings and faster time to generation compared to the generation and breeding of breeding genetically modified animals, this methodology is ideal for targets that are embryonic lethal, as it specifically targets the hematopoietic compartment. Furthermore, for targets where there are no tools available, such as an antibody, this system provides a feasible approach.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Eppendorf tube (line 2.2).

Eppendorf and Stemspan were removed in lieu of generic terms – centrifuge tube and serum free expansion media (SFEM). For example, as shown below, lines 148-149 on page 4.

Previous: 2.2 Puncture the bottom of 0.6 mL microcentrifuge tube with a 23 gauge needle and put it inside a 1.5 mL Eppendorf tube. Make 2 sets per animal.

Current: 2.2 Puncture the bottom of a 0.6 mL microcentrifuge tube with a 23-gauge needle and put it inside a 1.5 mL centrifuge tube. Make 2 sets per animal.

6. 1.1: when you refer to using 20% extra recipient mice, how many mice are optimal for this study?

We added the following lines to 1.1 (now 1.3 due to additional edits described in this response letter) which is specific to the CD40 agonistic antibody induced colitis model. Other models may

require different group sizes based on the models inherent variability and need for increased N to gain statistical power. Lines 135-139 on page 4.

Previous: 1.1 Design the experiment. For gene knockout (KO), it is recommended to use 3 – 6 gRNAs per gene. For each gRNA, prepare 20% extra recipient mice accounting for animal loss during and after transplantation.

Current: 1.3 Design the experiment. For gene knockout (KO), it is recommended to use 3 – 6 gRNAs per gene. For each gRNA, prepare 20% extra recipient mice accounting for animal loss during and after transplantation. The CD40 agonistic antibody induced colitis model requires a minimum of 8 mice per group for statistical powering, thus we made 10 mice per gRNA. The number of mice needed for other in vivo models will vary based on the model used.

7. 2.1: please specify the tools to be used to help readers/viewers replicate the protocol.

We substituted forceps and scissors for surgery tools - line 145 on page 4.

8. 2.3: Do you mean "put 4-8 pieces of bone"? Please specify if you prepare such sets for all the bones mentioned in 2.1, keeping the same type of bone in a given set of tubes.

We added the following lines to section 2.3 – lines 151-153 on page 4. At the stage, we have a tube full of bones from step 2.1, the resulting marrow is pooled, so we simply snip one end of every bone and fit however many bones we can into 1 microcentrifuge tube, the type of bone is of no concern.

Previous: 2.3 Cut one end of the bones open and put 4-8 bones inside the microcentrifuge tube with open ends facing towards the 23 gauge needle hole.

Current: 2.3 Cut one end of the bones open and put 4-8 bones inside the microcentrifuge tube with open ends facing towards the 23-gauge needle hole. The number of bones per tube depends on the bone, for example, 8 tibiae, humerus, and ulna or 4 femurs will fit.

9. 2.4 and 2.5: Do you centrifuge the outer 1.5 mL Eppendorf tube? Which tube do you discard in 2.5- the inner 0.6 mL tube? Do you add 1 mL of red blood cell lysis buffer to the outer 1.5 mL tube? What does the red blood cell lysis buffer contain?

The outer and inner centrifuge tubes are centrifuged together, such that the marrow is spun down into the bottom of the inner centrifuge tube, pulled through the hole made with the 23G needed made in step 2.2, and pelleted into the bottom of the outer centrifuge tube. Now the bones are empty, so the inner centrifuge tube can be discarded. RBC lysis buffer is then added to resuspend the pellet and remove RBC's. Lines 155-162 on page 4 were edited as shown below.

Previous: 2.4 Centrifuge with countertop centrifuge at 300 x g for 2 minutes at room temperature (RT).

2.5 Discard the microcentrifuge tube. Add 1 mL of red blood cell lysis buffer to each Eppendorf tube, and resuspend cell pellet by pipetting. Incubate at RT for 1 min. Repeat lysis step if needed.

Current: 2.4 Centrifuge the tubes (with the 0.6mL microcentrifuge tube containing the bones inside the 1.5mL centrifuge tube) using a countertop centrifuge at 300 x g for 2 minutes at room temperature (RT).

2.5 Discard the 0.6mL microcentrifuge tube, now containing bones with no marrow, leaving just the 1.5mL centrifuge tube now full of marrow. Add 1 mL of red blood cell lysis buffer (containing ammonium chloride) to each centrifuge tube and resuspend cell pellet by pipetting. Incubate at RT for 1 min. Repeat lysis step if needed.

10. 2.6: What volume conical tubes do you use? Please define DPBS at first use and specify its composition.

We added these details on lines 164-167 on page 4 as shown below.

Previous: 2.6 Transfer cell suspension into conical tubes and add DPBS to neutralize the lysis buffer. Pellet cells at 300 x g for 5 minutes. Aspirate supernatant completely.

Current: 2.6 Transfer the cell suspension into a 50mL conical tube and add Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (at least double the volume of the cell suspension) to neutralize the lysis buffer. Pellet cells at 300 x g for 5 minutes. Aspirate supernatant completely.

11. 2.7 and 6.2: Please change 70 µM filter to 70 µm filter.

Thank you – this error was corrected. Line 169 page 4 and line 272 on page 7.

12. 3.1: What is an optimal cell number (and hence, volume) to take?

Every cell that has been isolated thus far should be included. We updated section 3.1 as shown below to indicate that all of the cells counted from step 2.7 should be resuspended, and they should be resuspended at 90µL/10^7 cells. Lines 174-175 page 4.

Previous: 3.1 Re-suspend the cells in MACS buffer (DPBS, 2 mM EDTA, 0.5% BSA, 10 µg/mL PenStrep) in desired volume (90 µL per 10^7 cells).

Current: 3.1 Re-suspend the cells counted in step 2.7 in MACS buffer (DPBS, 2 mM EDTA, 0.5% BSA, 10 µg/mL PenStrep) in the desired volume (90 µL per 10^7 cells).

13. 4.4: Please use µg not ug.

Thank you – this error was corrected. Line 226 on page 5.

14. 4.6: What do you mean by “collect any extra cells remaining in the 96 well plate”?

Please see point 15 below for further explanation.

15. 4.6: Please rephrase to clarify “600 µL will be the volume per well in the 24 well plate following this step”. When do you use the 24-well plate?

This protocol utilizes a very small population of cells within the marrow. After collecting the 100µL from each well in the 96 well plate, we recommend washing the wells with medium to collect any of the precious LSK cells that remained in the plate. Thus, after collecting 5x100µL wells (each containing 10,000 LSKs) from the 96 well plate to get 50,000 LSK’s in each well of the 24 well plate, those 5 wells are then washed with 100µL of media, then added to the well with 500µL in the 24 well plate, resulting in a final volume of 600µL. We edited this section below to include more of these details for clarification. Lines 232-239 on page 6.

Previous: 4.6 Transfer 50,000 LSK cells in 500 µL medium to each Retronectin coated well. Use an additional 100 µL LSK medium per 5 wells to rinse and collect any extra cells remaining in the 96 well plate. 600 µL will be the volume per well in the 24 well plate following this step.

Current: 4.6 Transfer 50,000 LSK cells in 500 µL medium (5 wells from the 96 well plate in step 4.2) to each retronectin coated well on the 24-well polystyrene plate from step 4.4. Use an additional 100 µL LSK medium per 5 wells to rinse and collect any extra cells remaining in the 96 well plate (after transferring, look under a microscope to see there are cells remaining which weren’t transferred and can be collected using extra LSK medium to rinse and collect – this is a small population so you want to minimize cell loss). 600 µL will be the volume per well in the 24 well plate following this step (5x100 µL wells from the 96 well plate + 100 µL used to wash those 5 wells).

16. Note after 5.1: please specify or provide a reference to help readers/viewers understand how to perform the dose titration of their irradiator to identify the best dosage.

Another great point – we edited the note as shown below to include more details based on our experience as well as a reference to a great paper titled “Principals of bone marrow transplantation”. Lines 259-265 on pages 6-7

Previous: NOTE. The irradiation dosages may differ with different irradiators. It is recommended that users perform a dose titration of their irradiator to identify the best dosage.

Current: NOTE. The irradiation dosages may differ with different irradiators. It is recommended that users perform a dose titration of their irradiator to identify the best dosage. Doses between 700 and 1300 cGy for C57Bl/6 mice are found to be effective in various literature examples[17]. We recommend selecting 3-4 doses in this range and evaluating 5 mice per dose for survival and

engraftment. If engraftment is unsuccessful or the radiation dose is too high, mice will not live past 3 weeks post engraftment. At 4 weeks post engraftment, surviving mice can be bled to evaluate engraftment by FACS.

17. 6.1: Please reference the steps describing the harvesting of cells from the donor animals.

We changed this wording to indicate that the cells are coming from the wells in section 4.9 rather than harvesting from animals, and noted that at this point in the protocol your cells are no longer a large pool, since they have been transduced with different gRNA's, and should be kept separate. Lines 269-270 on page 7.

Previous: 6.1 Harvest the cells and pellet them at 300 x g for 10 minutes.

Current: 6.1 Pipet the cells out of each well from section 4.9, keeping groups separate now that the cells have been transduced with different gRNA's, and pellet them at 300 x g for 10.

18. 7.1: Before this, please add a note to describe the groups of mice in the study.

An additional note describing the experimental groups was added as shown below. Lines 287-291 on page 7.

NOTE: Experimental groups included – Naïve Control, Vehicle (Negative) Control, and Anti-p40 (Positive) Control groups: Together, these groups control for the normal behavior of the CD40 agonistic antibody induced colitis model. Vector, SgNone, and SgRNA groups: The vector controls for the common lentiviral vector, SgNone is a scrambled non-targeting guide control, and the SgRNA groups are the "treatment" groups bearing reduced expression of the target gRNA.

19. 7.2: As this is to be filmed, please specify how this is to be done.

Please see point 21 below for further explanation.

20. 7.2.2: How much enema and what is the composition of the enema to be given, please specify.

Please see point 21 below for further explanation.

21. 7.2.3: please change endoscopy to endoscope.

The sections below were edited to address points 19-21. Additional details were added to further describe video endoscopy, the enema is 1-2mL of DPBS, and typos were addressed. Lines 299-309 on pages 7-8.

Previous: 7.2.1 Anesthetize mice with 2-3% isoflurane.

7.2.2 Give mice DPBS enema to prepare the colon for endoscopy.

7.2.3 Insert endoscopy into the colon, advance to the proximal colon, then slowly withdraw and collect a video and images at 3cm, 2cm, and 1cm from the anus.

Current: 7.2.1 First, anesthetize the mice with 2-3% isoflurane.

7.2.2 Once under anesthesia, give mice DPBS (without calcium and magnesium) enema to prepare the colon for endoscopy. Approximately 1-2mL is required to perform an enema.

7.2.3 After enema and while under anesthesia, move a mouse to the anesthesia machine's nose cone.

7.2.4 Gently insert the endoscope into the colon, slowly advance to the proximal colon while keeping the camera centered and the colon inflated with air (using either the included air pump or attach a tube/syringe to manually inflate), then slowly withdraw the endoscope and collect a video and images at 3cm, 2cm, and 1cm from the anus.

22. 7.3: How much blood do you collect, from where? Please specify the method of euthanasia. Please provide references for histopathology (slide preparation, staining, magnification etc).

Section 7.3 was modified as shown below, including the recommended euthanasia method, blood collection, and a reference to a detailed description for histopathology. Lines 316-321 on page 8.

Previous: 7.3 Day 7: Euthanize all mice, collect blood for serum, weigh spleen and collect for flow cytometry, and collect colon for histopathology.

Current: 7.3 Day 7: Euthanize all mice by isoflurane overdose or CO_2 chamber, collect as much blood as possible by cardiac puncture for serum, weigh spleen and collect for flow cytometry, and collect colon for histopathology.

NOTE: Formalin-fixed and paraffin-embedded (FFPE) tissue sections of mouse colon were prepared and used for IHC as previously described by Wang et al[19].

23. Please do not abbreviate journal titles in the reference list.

Thank you - we edited the reference list to include full names of the journals.

24. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines:

Figure 1 legend (lines 289-291); Figure 3 (title and legend) lines 301-304 as these figure legends are not reused from a previous publication with permission.

We revised these sections as shown below (figures 1 and 3 are now 2 and 4 after adding new figure 1 schematic as suggested). Lines 362-370 and 379-383 on page 9.

Previous: Figure 1: Differentiation of immune cell subsets after LSK transplantation

LSK cells were isolated from CD45.2+ donor mice and transplanted to lethally irradiated CD45.1 congenic C57Bl/6 animals. Five mice were taken down every other week post-transplantation, and the engraftment rates in the spleen, bone marrow and blood were evaluated by FACS. Percent of donor cell subsets at different timepoints post-transplantation. Each dot represents a data point from a single animal.

Figure 3. Modulation of CD40 expression using a CRISPR/Cas9-based system

CRISPR/Cas9-mediated reduction of CD40 expression in the reconstituted mice. Splenic B cell CD40 expression was evaluated 8 weeks post-transplantation. Each dot represents an individual animal. *=P<0.005 Data shown are representative of two independent experiments.

Current: Figure 2: Differentiation of immune cell subsets after LSK transplantation

CD45.2+ donor mice were euthanized and their LSK cells were isolated then transplanted into lethally irradiated CD45.1 congenic C57Bl/6 animals. Every other week following transplantation, a small cohort of mice (n=5) were euthanized and the engraftment rates in the spleen, bone marrow, and blood were evaluated by FACS. Percent of donor cell subsets at different timepoints post-transplantation is shown. Each dot represents a data point from a single animal.

Figure 4. CRISPR/Cas9-mediated reduction of CD40 expression on B-cells

*CD40 expression reduction in reconstituted mice, modulated using a CRISPR/Cas9-based platform. Splenocytes were evaluated 8 weeks post transplantation by FACS for CD40 expression on B-cells. Each dot represents an individual animal. *=p<0.005 Data shown are representative of two independent experiments.*

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26. Figures 2, 4, and 5: Please make the dimensions under the scale bar visible.

Thank you for pointing this out, this edit has been made on all 3 figures.

Reviewer #1:

Manuscript Summary:

In the present methods article, Sean Graham and Rui Wang they have develop a platform in which potential target genes involved in IBD pathogenesis can be rapidly knock out to assess their potential therapeutic effect. To do so, they use Cas9 expressing mice to isolate LSK population from their bone marrow, which they later manipulate in vitro to knock out the gene of interest (in the example is CD40). Finally, they transplant these KO-LSK cells into WT recipient mice treated with and anti-CD40 agonist (to induce inflammation and recapitulate IBD pathogenesis). Although this procedure could be used to provide valuable information of candidate genes involved in IBD, I believe it could be better explained in order other investigators could follow the steps. Below are my comments that could help to improve understanding of the present article.

Major Concerns:

- In the introduction, before going into the details of the protocol I would write a paragraph where it is explained the entire procedure maybe together with a schematic picture. (i.e "The protocol consists of isolation of LSK cells from bone marrow from Cas9 mice etc…")

Thank you again for this great suggestion. The following text has been added to the introduction and a new Figure 1 has been added with the following title and legend. Introduction text: Lines 85-96 on pages 2-3. Figure 1 title/legend: Lines 362-370 on page 9.

The protocol presented here provides viable alternative to breeding genetically modified mice. First, as shown in the Figure 1 schematic, Lineage-Sca1+c-Kit+ (LSK) cells are isolated from the bone marrow of Cas9 KI mice bearing a specific allele (CD45.2) to allow donor immune cell tracking. Next, these cells are exposed to lentiviruses bearing different gRNAs and a fluorescent marker (VexGFP) to allow tracking of transduced cells. Two days later, VexGFP+ cells are sorted and injected into lethally irradiated recipient Ly5.1 Pepboy mice, which are C57Bl/6 mice bearing the CD45.1 allele to allow for recipient immune cell tracking. 12 weeks later, the immune system is fully reconstituted and the mice can be enrolled into in vivo models. In

addition to the benefit of cost savings and faster time to generation compared to the generation and breeding of breeding genetically modified animals, this methodology is ideal for targets that are embryonic lethal, as it specifically targets the hematopoietic compartment. Furthermore, for targets where there are no tools available, such as an antibody, this system provides a feasible approach.

Figure 1: Stem cell harvest, transduction, and transplantation strategy used to generate CRISPR/Cas9-edited mice

Bones are harvested from Cas9 KI mice, bearing the CD45.2 allele as a donor cell marker. Bone marrow is the isolated and the cells are sorted for Lin-Sca1+C-Kit+ (LSK) stem cells. These cells are then exposed to various lentiviruses (vector control, SgNone Control, SgCD40.1, etc) all bearing VexGFP as a fluorescent marker to indicate cells which were transduced. Stem cells are then sorted for VexGFP+ cells to inject a pure population of edited stem cells into lethally irradiated recipient mice. The recipient mice are Ly5.1 pepboy mice, which are C57Bl/6 WT mice bearing the CD45.1 allele as a recipient cell marker.

- In general, all titles are short, they should provide more information. For instance, I am assuming you have knocked out CD40 in LSK cells, but it is not said in the title or in the text when this specific part of the protocol is described.

The titles have been updated, for example section 4 as noted below, now indicates that in this step the knockout cells will be generated. Line 217 on page 4.

Previous: 4. LSK infection and culture

Current: 4. LSK transduction and culture to generate control and knockout cells

- The LSK transduction section it is poorly described. I am missing fundamental information of how the virus was generated (in which construct are sgRNAs cloned into? Did you clone the sgRNAs or the construct was purchased), Are virus titrated? The only information I could find is that a pLentivirus construct was used. If this platform wants to be used to KO other genes of interest as it is point out in the discussion, it is important to explain in the protocol how to clone sgRNAs in the constructs (cloning strategy).

Thank you for bringing this gap to our attention. We have modified section 1 of the protocol to include the following details. Lines 116-127 on page 3.

1.1 The plasmids were contructed using a lentiGuide-puro vector, which was modified to VexGFP or mCherry. The scrambled non-targeting gRNA (SgNone) or CD40 targeting gRNA's were then cloned into the modified vector. The sequences for each gRNA were: SgNone, CTATGATTGCAACTGTGCAG; SgCD40.1, AGCGAATCTCCCTGTTCCAC; SgCD40.2, GACAAACAGTACCTCCACGA; SgCD40.3, ACGTAACACACTGCCCTAGA. Lentiviral particles were then produced as previously described[15]. First, the gRNA encoding plasmids were

co-transfected into 293T cells with VSV-G and pLEX packaging plasmids. The culture medium was changed after 18 hours of transfection and the supernatants containing the virus were collected after 24-48 hours following the medium change. To evaluate CD40 gRNA efficiency, a CD40 stable 293T cell line was generated by transfection of a pcDNA3.1 plasmid that encodes for CD40. The cells were then transfected using the described lentiviruses and evaluated by FACS two weeks later.

Minor Concerns:
- In lines 94-95 where the mice used are explained, it is writing in a confusing manner. The donor mice are Cas9KI/CD45.2+ and the recipient are WT mice (both of them C57Bl/6 background)?

Thank you for this comment, we hope the added text shown below clarifies the purpose of using strains which are both on the C57Bl/6 background. We use "pepboy" mice which are expressing CD45.1 on all their immune cells, but other than that they are standard WT C57Bl/6 mice. These are the mice we transplant Cas9 KI cells also from a C57Bl/6 background so there will be no GvHD. Since the Cas9 KI cells have CD45.2 on all their immune cells, we can then evaluate engraftment by comparing CD45.2 vs CD45.1 cells in the recipient mouse. Lines 129-133 on pages 3-4.

Previous: Cas9 knock-in (KI) mice generated by Dr. Feng Zhang's lab were used as donor mice, purchased on license from Jackson Laboratories. Cas9 KI mice generated by other groups may be suitable as well, provided they are on a C57Bl/6 background when using C57Bl/6-Ly5.1 pepboy mice as recipients.

Current: 1.2 Cas9 knock-in (KI) mice generated by Dr. Feng Zhang's lab were used as donor mice, purchased on license from Jackson Laboratories. Cas9 KI mice generated by other groups may be suitable as well, provided they are on a C57Bl/6 background (and are expressing the CD45.2 allele) when using C57Bl/6-Ly5.1 pepboy mice (expressing the CD45.1 allele) as recipients, so donor and recipient cells can be tracked.

- Write down what LSK stands for: Lin-Sca1+CD117. First time mention in line 127

We made this change – thank you for pointing it out! Line 86 on page 2.

- I don't personally like the term infection, it is better transduction (Line 168)

We changed infection to transduction throughout – beginning on line 89 on page 2.

- Provide the sequence of sgRNA used.

The sequences were added to section 1 of the protocol. Lines 118-120 on page 3.

The sequences for each gRNA were: SgNone, CTATGATTGCAACTGTGCAG; SgCD40.1, AGCGAATCTCCCTGTTCCAC; SgCD40.2, GACAAACAGTACCTCCACGA; SgCD40.3, ACGTAACACACTGCCCTAGA.

- Was it ever tested the CD40 KO efficiency in LSK cells before transplantation in recipient mice?

The following text was added to the beginning of the protocol. Lines 124-127 on page 3.

To evaluate CD40 gRNA efficiency, a CD40 stable 293T cell line was generated by transfection of a pcDNA3.1 plasmid that encodes for CD40. The cells were then transfected using the described lentiviruses and evaluated by FACS two weeks later.

Reviewer #2:

Manuscript Summary:

In this manuscript, the authors developed a method to study gene function in vivo by rapidly generating CRISPR-based gene knockout mouse models. It is a well written manuscript with solid data. Therefore, the manuscript is suitable for publication in JOVE. However, following issues need to be addressed before accepting for publication.

Minor Concerns:

1. The experimental descriptions were not presented in detail. More details need to be supplemented.

Additional details have been added throughout the protocol. Many of the needed details have been pointed out by the reviewers and addressed throughout this response letter. We thank the reviewers for pointing this out and ultimately, improving our written protocol.

1) In Step 2 and 3 of this protocol, it would be helpful to readers if the authors could provide the general yield of target cells (eg. CD117+ cells and LSK cells) per donor mouse in each step.

We added expected numbers based on a donor cohort of 30 animals, which is our standard donor cohort size, to each step where cell counting is mentioned - sections 2.7, 3.9, and 3.13. Lines 169-171 on page 4, lines 198-200 on page 5, and lines 209-210 on page 5.

2) In Step 4, details about lentivirus production or concentration should be provided.

An additional note regarding the preferred number of viral particles per mL was added as shown below. Lines 241-243 on page 6.

Previous: 4.7 Add 300 µL virus supernatant on top of each well and shake the plate at 500 SV for 5min. Spin the plate at 600 x g and 37 °C for 20 minutes.

Current: 4.7 Add 300 µL virus supernatant on top of each well and shake the plate at 500 SV for 5min. Spin the plate at 600 x g and 37 °C for 20 minutes. We recommend using virus with at least 5x10^6 viral particles per mL.

Furthermore, additional details were added in section 1.1 as noted above and shown below. Lines 116-127 on page 3.

1.1 The plasmids were contructed using a lentiGuide-puro vector, which was modified to VexGFP or mCherry. The scrambled non-targeting gRNA (SgNone) or CD40 targeting gRNA's were then cloned into the modified vector. The sequences for each gRNA were: SgNone, CTATGATTGCAACTGTGCAG; SgCD40.1, AGCGAATCTCCCTGTTCCAC; SgCD40.2, GACAAACAGTACCTCCACGA; SgCD40.3, ACGTAACACACTGCCCTAGA. Lentiviral particles were then produced as previously described[15]. First, the gRNA encoding plasmids were co-transfected into 293T cells with VSV-G and pLEX packaging plasmids. The culture medium was changed after 18 hours of transfection and the supernatants containing the virus were collected after 24-48 hours following the medium change. To evaluate CD40 gRNA efficiency, a CD40 stable 293T cell line was generated by transfection of a pcDNA3.1 plasmid that encodes for CD40. The cells were then transfected using the described lentiviruses and evaluated by FACS two weeks later.

3) In Step 6, results of lentiviral transduction rate indicated by VexGFP should be supplemented.

The expected transduction rate was added as shown below. Lines 272-273 on page 7.

Previous: 6.2 Resuspend the cells in MACS buffer, filter with a 70 µM strainer, and FACS sort VexGFP+ population.

Current: 6.2 Resuspend the cells in MACS buffer, filter with a 70 µM strainer, and FACS sort VexGFP+ population. You should expect 10-15% of the cells to be VexGFP+.

2. In Fig5d, CD86 can be used as a marker for B cell activation, while it may not be a good marker for T cell activation, other markers like CD44 or CD69 should be used, unless the authors could provide some relevant references.

Thank you for this comment. For any future experiments we will consider using CD44 and CD69 as T-cell activation markers. Please see the reference below as evidence that CD86 can also be used as a T-cell marker, more specifically for recent activation. However as previously noted your point is well taken and we will further evaluate these markers.

Discovery of CD80 and CD86 as recent activation markers on regulatory T cells by protein-RNA single-cell analysis

Dominik Trzupek, Melanie Dunstan, Antony
J. Cutler, Mercede Lee, Leila Godfrey, Dominik Aschenbrenner, Holm H. Uhlig, Linda
S. Wicker, John A. Todd, Ricardo C. Ferreira
bioRxiv 706275; doi: https://doi.org/10.1101/706275

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